Evidence for a Two-State Transition in the Folding Process of the Activation Domain of Human Procarboxypeptidase A2†

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ABSTRACT: The activation domain of human procarboxypeptidase A2 (ADA2h), a globular open-sandwich $\alpha + \beta$ domain with 80 residues and no disulfide bridges, has been studied by thermodynamic and kinetic analysis. Equilibrium denaturation by urea or temperature is fully reversible at pH 7.0 and fits to a two-state transition. The Gibbs energy of unfolding extrapolated to null concentration of chemical denaturant, ΔG_{H_2O}, at pH 7.0 and 298 K, is calculated to be 17.0 ± 1 kJ mol^{-1}, which is within experimental error of the value determined by differential scanning calorimetry, 15.1 ± 2 kJ mol^{-1}. Kinetics of unfolding and refolding followed by fluorescence do not show the presence of any kinetic intermediate accumulating in the folding reaction. A value for ΔG_{H_2O} of 17.9 ± 0.7 kJ mol^{-1} can be extrapolated from the kinetic data. All these data indicate that the folding pathway of this domain is consistent with a two-state model (with the exception of the *cis*-Pro intermediates). More importantly, the analysis of this and several other small domains or proteins supports the hypothesis that stable kinetic folding intermediates are not necessary for a protein to fold. There seems to be a relationship between the size of a protein and the presence of stable kinetic intermediates. Globular proteins with less than 80 residues and no disulfide bonds follow a two-state transition, while proteins larger than 100 residues present stable kinetic folding intermediates.

The detailed mechanism by which proteins acquire their specific, biologically active conformation from the information contained in the amino acid sequence is one of the central unsolved problems of molecular biology (Anfinsen & Scheraga, 1975; Creighton, 1992). It is generally accepted that folding of a polypeptide chain cannot occur by an overall random-search mechanism, but rather there must be one or more folding pathways that restrict the conformational search. Kinetic and/or equilibrium studies of many different proteins with more than 100 residues (Ikeguchi *et al.*, 1986; Kuwajima *et al.*, 1988; Griko *et al.*, 1988; Udgaonkar & Baldwin, 1990; Briggs & Roder, 1992; Lu & Dahlquist, 1992; Radford *et al.*, 1992; Serrano *et al.*, 1992; Varley *et al.*, 1993; Mann & Matthews, 1993; Filimonov *et al.*, 1993; Muñoz *et al.*, 1994) have indicated the existence of intermediates. These intermediates have been postulated to be important for protein folding and to represent some of the steps leading from the unfolded to the folded state (Serrano *et al.*, 1992). However, there are cases for which neither an equilibrium nor a kinetic intermediate has been detected using the standard set of techniques: the chymotrypsin inhibitor CI2 (Jackson & Fersht, 1991; Otzen *et al.*, 1994), the B1 domain of the IgG-binding protein (Alexander *et al.*, 1992a,b), the α-spectrin SH3-domain (Viguera *et al.*, 1994), the acyl-coenzyme A binding protein (Kvagelund *et al.*, 1995), and the CspB protein (Schindler *et al.*, 1995). The acyl-coenzyme A binding protein is an 86 aa all α-protein (Andersen & Poulsen, 1992). The CI2 protein inhibitor has 80 amino acids, but only 60 of them are contained within a globular domain (McPhalen & James, 1987). The B1 domain of the IgG-binding protein contains 57 residues (Fahnestock *et al.*, 1986). In both cases, there is a central α-helix packing against an antiparallel β-strand (McPhalen & James, 1987; Lian *et al.*, 1991; Gronenborn *et al.*, 1991; Orban *et al.*, 1992). On the other hand, the SH3-domain of α-spectrin (Musacchio *et al.*, 1992a,b) and the CspB protein (Schnuchel *et al.*, 1993; Schindelin *et al.*, 1993) contain around 60 residues and consist almost exclusively of β-structure. The common feature among all these cases is the small size of the polypeptide being studied. This raises the following question: Are the intermediates accumulating during the refolding of other proteins the result of size and, consequently, of complexity? If this is true, then it should be expected that other small proteins or domains, with no disulfide bridges, should also lack any populated intermediate and behave as pure two-state systems (Viguera *et al.*, 1994).

Procarboxypeptidases are activated when trypsin produces a single cleavage at the boundary between the activation segment and the carboxypeptidase region; subsequently, a definitive sequence of cleavages occurs at the C-terminal of the relased activation segment giving rise to several intermediary fragments and, finally, to a stable N-terminal globular peptide known as activation domain (Vendrell *et al.*, 1990; Burgos *et al.*, 1991; Villegas *et al.*, 1995). The three-dimensional structures of this piece in different forms of procarboxypeptidases (A1, A2, and B) have been derived, either in the isolated state (Vendrell *et al.*, 1991) or within the proenzyme (Coll *et al.*, 1991; Guasch *et al.*, 1992, Catasús *et al.*, 1995). These structures are highly conserved, consist-

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ing mainly of a globular region with two α-helices and four β-strands arranged in an open-sandwich antiparallel α-antiparallel β topology. The recombinant activation domain of human pancreatic procarboxypeptidase A2 (ADA2h)[1] is an 80-residue domain with no disulfide bridges and 4 *trans*-prolyl bonds. This domain is an interesting subject of study, not only because its size is midway between those proteins presenting folding intermediates (>110 residues) and those following a two-state mechanism (~60 residues) but also because it could play an important role in the folding of the complete proenzyme.

In this work, the thermodynamic and kinetic behavior of ADA2h has been analyzed. We have used urea to denature the protein, and measured the changes in fluorescence to follow the unfolding process. Differential scanning calorimetry experiments were also performed with the double aim of establishing the thermodynamic parameters and checking the fitting to the two-state approximation by another complementary metodology. Kinetic analysis of the unfolding and refolding reactions, using urea concentration jumps, was performed to thoroughly check the presence of any intermediate not detected under equilibrium conditions.

EXPERIMENTAL PROCEDURES

Chemicals. Guanidine hydrochloride (Gdn·HCl) and urea were purchased from BRL (Gaithersburg, MD). The buffers for equilibrium denaturation were sodium phosphate and sodium pyrophosphate from Merck (Darmstadt, Germany). All other reagents were of the highest purity available. Double-distilled deionized water was used throughout.

Expression and Purification of the Recombinant ADA2h. The plasmid pIN-III-*ADA2h* containing the native ADA2h was expressed in *Escherichia coli* MC1061. Bacteria were grown in M9CAS at 37 °C from a single colony until an absorbance at 600 nm of ~0.4 was reached. Isopropyl β-D-thiogalactopyranoside was then added to a final concentration of 0.2 mM, and the culture was grown overnight at 37 °C. Cells were discarded by centrifugation, and the supernatant was concentrated by tangential filtration. The concentrated supernatant was loaded onto a SEP-PAK VAC C18 Cartridge (Waters Millipore, Co.), extensively washed with 25% acetonitrile, and eluted with 30% 2-propanol. After freeze-drying, the sample was loaded onto a TSK-DEAE 5PW column (150 × 21.5 mm, 10 μm particle-size, 0.1 μm pore, from Toyoshoda) in 25 mM Tris−acetate (pH 7.5), and an elution gradient between 0 and 0.8 M ammonium acetate was used. As a last step, desalting by a Sepharose G-25 column was included.

Protein Concentration Determination. The extinction coefficient was calculated by the method of Gill and von Hippel (1989), based on the measurement of absorbance at 280 nm of a protein preparation in 6 M Gdn·HCl and its comparison with the value obtained for a native protein preparation. A molar extinction coefficient of 6970 was ascribed to the denatured protein since only one tryptophan and one tyrosine contribute to this signal in ADA2h. The following equation was used:

$$\epsilon_{M,Nat} = A_{Nat}\epsilon_{M,Gdn\cdot HCl}/A_{Gdn\cdot HCl}$$

where $\epsilon_{M,Nat}$ is the molar extinction coefficient and A_{Nat} is the absorbance of the native protein. $\epsilon_{M,Gdn\cdot HCl}$ and $A_{Gdn\cdot HCl}$ are the molar extinction coefficient and the absorbance of the denatured protein, respectively. The extinction coefficient at 280 nm for a 1 mg/mL native ADA2h solution in an 1 cm path cell is calculated to be 0.78.

Chemical Denaturation Experiments. Urea and Gdn·HCl solutions were prepared gravimetrically in volumetric flasks. For each data point, 100 μL of ADA2h in sodium phosphate (pH 7.0) was mixed with 750 μL of a given denaturant solution, rendering a final buffer concentration of 50 mM. The mixtures of the protein plus the buffer in the appropiate denaturing solutions were left to equilibrate for 1−24 h, and each data point was assayed 4 times.

Fluorescence Spectroscopy Analysis. Fluorescence emission spectra of tryptophan 38 of ADA2h were used to monitor any changes in the environment of this residue upon the unfolding of the protein. Fluorescence was measured in an Aminco Bowman Series 2 luminescence spectrometer. Excitation was at 290 nm with a 2 nm slit width. Fluorescence was detected through an 8 nm slit width. In these experiments, protein concentration was kept at 2.2 μM, and temperature at 298 K. The fluorescence, corrected for the instrument response of ADA2h, is reduced in intensity and red-shifted from 348 to 352 nm upon denaturation with urea or guanidine hydrochloride (data not shown). We found the maximum difference between the native and the denatured states to be centered at 339 nm; however, we chose 315 nm to monitor protein denaturation in order to minimize the urea contribution to the signal. The equilibrium constant for denaturation can be calculated, for each denaturant concentration, by using eq 1:

$$K_U = (F_N - F)/(F - F_U) \quad (1)$$

where F is the fluorescence value at a certain concentration of denaturant and F_N and F_U are the corresponding fluorescence values for the fully folded and unfolded states, respectively. It has been found experimentally that the free energy of unfolding of proteins in the presence of urea is linearly related to the concentration of denaturant (Pace, 1986):

$$\Delta G_U = \Delta G_{H_2O} - m[\text{denaturant}] \quad (2)$$

The values of m and ΔG_{H_2O}, the apparent free energy of unfolding in the absence of the denaturant, may be calculated from eq 1, because $\Delta G_U = -RT \ln K_U$. The proportionality constant m reflects the cooperativity of the transition and is believed to be related to the difference in the hydrophobic surface exposed to the solvent between the native and the denatured states.

The quantum yield of the fluorescence of the native and denatured protein increases with urea concentration (Figure 1). Taking all of these dependences into account, the fluorescence data can be fitted to the equation:

$$F = \{(F_N + a[\text{urea}]) + (F_U + b[\text{urea}]) \exp(m[\text{denat}] - \Delta G_{H_2O})/RT\}/\{1 + \exp(m[\text{denat}] - \Delta G_{H_2O})/RT\} \quad (3)$$

in which the dependence of the intrinsic fluorescence upon denaturant concentrations, in both the native and the denatured states, is taken into account by the terms a[urea] and

[1] Abbreviations: ADA2h, Activation domain of human procarboxypeptidase A2; Gdn·HCl, guanidine hydrochloride; CD, circular dichroism; DSC, differential scanning calorimetry.

b[urea], respectively (linear approximation). With this kind of analysis, a two-state model for denaturation is being assumed, with no species other than the native and denatured forms of the domain accumulating significantly.

The values obtained for ΔG_{H_2O} and m are only reliable when both a and b can be calculated accurately, that is, when the total transition can be observed and several values of the fluorescence of the native and denatured states over a large range of denaturant concentrations are obtained.

Circular Dichroism. CD equilibrium measurements were performed in a Jobin-Yvon C-VI machine. Far-UV circular dichroism spectra were recorded in a 0.2 cm path length cell at 298 K.

Differential Scanning Microcalorimetry. Scanning microcalorimetry was performed in a computerized version of the DASM-4 microcalorimeter (cell volume 0.47 mL; Privalov & Potekhin, 1986) at a heating rate of 2 K/min with protein concentrations of about 2 mg/mL. Before being placed into the cell, the samples were extensively dialyzed againts buffers with the appropriate pH. The buffers used for the calorimetric experiments were 50 mM or 5 mM sodium phosphate, both at pH 7.0, and 50 mM or 5 mM sodium pyrophosphate at either pH 8.0 or pH 9.0. A constant pressure of 1.5 atm was maintained during all DSC experiments to prevent possible degassing of the solutions upon heating. The reversibility of the thermally induced transitions was always checked by reheating the solution in the calorimeter cell immediately after cooling from the first run. Base lines, obtained by filling both cells with the corresponding buffer for each particular condition, were subtracted from the sample experimental trace. To calculate the molar partial heat capacity of the domain from DSC data, we used a partial specific volume of 0.73 mL/g, the average value for small globular proteins (Privalov & Kechinashvili, 1974).

In all calculations, the molecular mass of the domain deduced from its sequence (Catasús *et al.*, 1995) and corroborated by mass spectrometry (result not shown) was taken as 9212. The calorimetric data were analyzed as described by Viguera *et al.* (1994).

Kinetic Analysis. Kinetics were followed in a Bio-Logic stopped-flow machine (SFM-3) by fluorescence. The average dead time of the experiments was 50 ms due to artifacts arising from mixing water and high urea concentations. A cell of 150 μL and an aging loop of 10 μL were used. The unfolding reaction was performed by dilution of the native ADA2h in 50 mM sodium phosphate buffer (pH 7.0) with the appropiate ratio of the same buffer containing different concentrations of urea. For the refolding reaction, the unfolded domain in 50 mM sodium phosphate buffer (pH 7.0), containing 7.5 and 9 M urea, was mixed with an excess of the same buffer without urea to give several final urea concentrations. Fluorescence was measured through a 320 nm cutoff filter (excitation at 290 nm). The cell chamber and the syringes were kept at 298 K.

RESULTS

Equilibrium Chemical Denaturation Experiments. The chemical denaturation by urea of ADA2h followed by fluorescence is shown in Figure 1. Denaturation of this domain is cooperative and can be fitted to a two-state denaturation model. At pH 7.0 (in 50 mM sodium phosphate) and 298 K, the calculated values for ΔG_{H_2O} and m are 17.0 ± 1 kJ mol^{-1} and 4.0 ± 0.2 kJ mol^{-1} M^{-1},

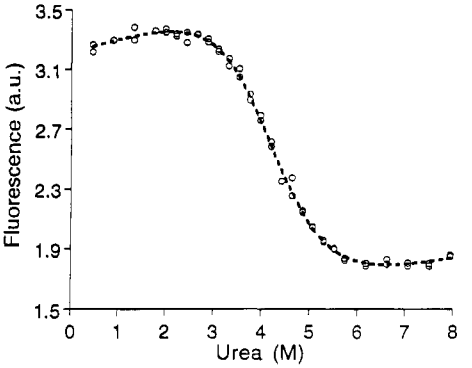

FIGURE 1: Chemical denaturation profile by urea of the activation domain of human procarboxypeptidase A2 (ADA2h) followed by fluorescence in 50 mM sodium phosphate at pH 7.0. The dataset, resulting from four different experiments, was fitted to eq 3.

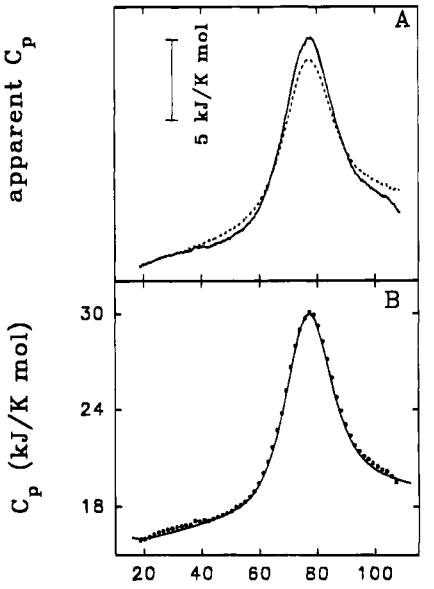

FIGURE 2: (A) Original calorimetric recordings, after subtracting the instrumental base line, for ADA2h in 50 mM sodium phosphate at pH 7.0. (—) first scanning of the sample; (•••) second scanning of the same sample. (B) Best fitting of the experimental temperature dependence of the partial molar heat capacity data (circles) of the domain in 50 mM sodium phosphate, pH 7.0, to the two-state model (solid line).

respectively. Slope m is slightly higher than that observed for the SH3 domain of α-spectrin (~3.2 kJ mol^{-1} M^{-1}; Viguera *et al.*, 1994), as expected from its larger size. The denaturation inflection point is about 4.2 M urea. When the protein is denatured, as monitored by fluorescence, there is a concomitant loss of secondary structure shown by far-UV CD analysis (data not shown).

Differential Scanning Microcalorimetry. The thermal unfolding of the ADA2h domain is reversible upon reheating the sample, the reversibility being highest at pH 7.0, 50 mM phosphate (Figure 2A). The recovery of the endotherm upon reheating decreases to some extent both at lower ionic strength (5 mM) and at higher pH values (8 and 9). The thermal transition at pH 7.0, 50 mM phosphate, fits the two-state equilibrium model very well, with an equal value for both the calorimetric and van't Hoff enthalpies within experimental uncertainty. Figure 2B shows the excellent fitting of the data to the equations of a two-state model (Viguera *et al.*, 1994). On decreasing the ionic strength, or increasing the pH, the fitting of the DSC data to a monomolecular two-state model is progressively less ad-

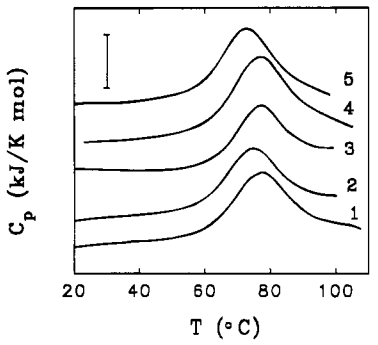

FIGURE 3: Temperature dependence of the molar heat capacity for the activation domain ADA2h under different conditions: (1) 50 mM phosphate, pH 7.0; (2) 5 mM phosphate, pH 7.0; (3) 50 mM pyrophosphate, pH 8.0; (4) 50 mM pyrophosphate, pH 9.0; (5) 5 mM pyrophosphate, pH 9.0. The bar shown corresponds to 10 kJ K^{-1} mol^{-1}.

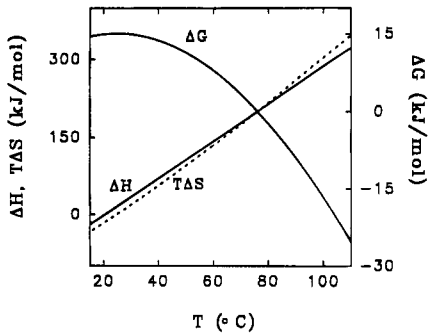

FIGURE 4: Temperature dependence of ΔH, $T\Delta S$, and ΔG functions for the thermal unfolding of ADA2h in 50 mM phosphate, pH 7.0.

equate. Thus, at either pH 8.0, 50 mM pyrophosphate, or pH 7.0, 5 mM phosphate, the calorimetric to the van't Hoff enthalpy ratio is 0.9, whereas at pH 9.0, 5 mM pyrophosphate this ratio is 0.73. This indicates that at lower ionic strength and higher pH values there are intermolecular interactions giving rise to an association/dissociation process for the folded domain coupled to its thermal unfolding (Filimonov *et al.*, 1993).

The T_m values for ADA2h are rather high, ranging from 70 to 77 °C in the studied conditions. This high thermal stability and its decrease with increasing pH (Figure 3) are similar to those found for the activation domain of both porcine procarboxypeptidases A (Sánchez-Ruiz *et al.*, 1988) and B (Conejero-Lara et *al.*, 1991). The melting temperature and the unfolding enthalpy of ADA2h at pH 7.0 and 50 mM phosphate are 77 °C and 199 kJ mol^{-1}, respectively.

The average specific values of the C_p value at 20 °C, 16.0 ± 1.6 kJ K^{-1} mol^{-1}, the predenaturational C_p slope, 0.07 ± 0.01 kJ K^{-2} mol^{-1}, and the heat capacity change upon unfolding, 3.6 ± 1.4 kJ K^{-1} mol^{-1}, are close to those described for small compact globular proteins (Privalov & Kechinashvili, 1974; Privalov, 1979). Using the above-mentioned average ΔC_p of unfolding, we have obtained the unfolding entropy, enthalpy, and Gibbs energy changes, at pH 7.0, 50 mM phosphate, as a function of temperature, as shown in Figure 4. The ΔG of unfolding at 298 K is 15.1 ± 2 kJ K^{-1} mol^{-1}, similar to the corresponding ΔG value found for the activation domain of porcine procarboxypeptidase B, 13.4 kJ K^{-1} mol^{-1} (Conejero-Lara *et al.*, 1991).

Kinetic Analysis of the Refolding and Unfolding Reactions. It has been reported that the presence of any intermediate is unequivocally shown by the kinetic analysis of the refolding reaction, including many proteins for which no intermediate

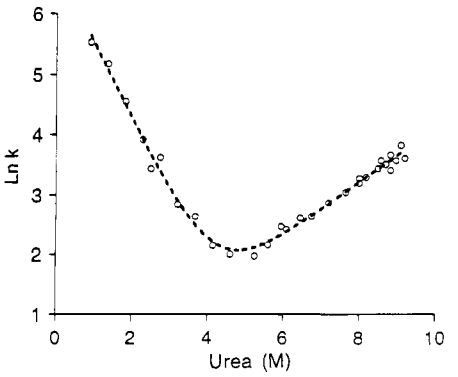

FIGURE 5: Kinetics of the refolding and unfolding reactions of the ADA2h domain followed by fluorescence and urea perturbation in 50 mM sodium phosphate at pH 7.0. The solid line is the best fit of the whole dataset to eq 4.

is found under equilibrium conditions (Matouschek *et al.*, 1992). To check whether a kinetic intermediate could be detected in the refolding of ADA2h, a kinetic analysis under the same conditions used in equilibrium was performed. The result of this analysis is shown in Figure 5. There is a linear dependence of the logarithm of the rate constant of ADA2h unfolding (k_u), with urea concentration, in agreement with the known fact that in an unfolding reaction the strong denaturation conditions normally prevent the accumulation of any kinetic intermediate (Matouschek *et al.*, 1992). In the refolding reaction, two main transitions are observed: a fast transition (k_f) completed in less than 0.2 s, and a slow one, independent of the urea concentration, with a kinetic constant of 0.021 ± 0.007 s^{-1} (k_f'; data not shown). The logarithm of k_f *vs* urea can be fitted to a linear equation, outside the transition region. The whole reaction can be fitted to the equation (Jackson & Fersht, 1991):

$$\ln k = \ln[k_{f,H_2O} \exp(-m_{k_f}[\text{urea}]) + k_{u,H_2O} \exp(-m_{ku}[\text{urea}])] \quad (4)$$

where k is the rate constant at a given concentration of denaturant, k_{f,H_2O} is the rate constant of refolding in water, k_{u,H_2O} is the rate constant of unfolding in water, and m_{k_f} and m_{k_u} are the slopes of the refolding and unfolding reactions, respectively. The kinetic parameters calculated from this equation are as follows: k_{f,H_2O} = 897 ± 107 s^{-1}, k_{u,H_2O} = 0.65 ± 0.12 s^{-1}, m_{k_f} = 5.28 ± 0.21 kJ mol^{-1} M^{-1}, and m_{k_u} = −1.84 ± 0.08 kJ mol^{-1} M^{-1}. The rate constants of refolding and unfolding of ADA2h in water are very high when compared with the same constants for other small proteins described in the literature, for instance, the rate constants obtained for the SH3-domain of α-spectrin (Viguera *et al.*, 1994) or CI2 (Jackson & Fersht, 1991), but are similar to that of CspB (Schindler et al., 1995). In the native state, ADA2h contains four *trans*-prolyl bonds. The slow rate constant which is independent of urea concentration, k_f', can be the result of the *cis-trans* isomerization of the prolyl bonds in the protein. The fact that we can only distinguish one slow rate constant indicates either that all the prolines isomerize with similar rate constants or that some of the *trans* bonds are not necessary for the protein to fold correctly.

The values of the free energy of unfolding ΔG_{H_2O} and slope m, which reflect the cooperativity of the transition, can be estimated from the above parameters, taking into account the equilibrium:

$$U_c \overset{k_{iso}}{\leftrightarrow} U_t \overset{k_{f,H_2O}}{\underset{k_{u,H_2O}}{\leftrightarrow}} N \quad (5)$$

k_{iso} is defined as the ratio between the amount of protein in the *cis* and *trans* conformations in the unfolded state. When the native conformation of the proline residues of a protein is in a *trans* conformation, the contribution of the *cis-trans* isomerization to the free energy of unfolding is very small and can be considered negligible (Jackson & Fersht, 1991). Thus, a reasonable estimation of ΔG_{H_2O} from the second equilibrium can be obtained by using

$$\Delta G_{H_2O} = -RT \ln(k_{f,H_2O}/k_{u,H_2O}) \quad (6)$$

and slope m can be obtained from eq 7:

$$m = RT(m_{k_f} - m_{k_u}) \quad (7)$$

Using eqs 6 and 7, we obtain the following values: $\Delta G_{H_2O} = 17.9 \pm 0.7$ kJ mol^{-1} and $m = 4.22 \pm 0.3$ kJ mol^{-1} M^{-1}, which are very similar to those found under equilibrium conditions.

DISCUSSION

Thermodynamic Stability of ADA2h. The free energy of unfolding of ADA2h at pH 7.0 and 298 K is around 17 kJ mol^{-1} with an m value of around 4.0 kJ mol^{-1} M^{-1}, as has been assayed by equilibrium, kinetic, and calorimetric analyses. The former value is low compared with the stability of small globular proteins of similar size (25−29 kJ mol^{-1} at 298 K; Jackson & Fersht, 1991; Alexander *et al.*, 1992a). However, it is very close to that of the activation domain of procarboxypeptidase B (Conejero-Lara *et al.*, 1991) and, more interestingly, to that found for another small globular domain, the SH3-domain of α-spectrin (~16 kJ mol^{-1}; Viguera *et al.*, 1994). The smaller values obtained for globular domains, compared to proteins of similar size, are not so surprising since it is reasonable to think that interactions with other domains in the protein, for example, the hydrogen bonds established with the active site of the enzyme in ADA2h (Catasús *et al.*, 1995), could stabilize them. From the calorimetric point of view, ADA2h seems to be an ordinary example of a small single-domain protein; the thermodynamic parameters are all close to those described for small compact globular proteins (Privalov & Kechinashvili, 1974; Privalov, 1979). Thus, for all intents and purposes, ADA2h behaves in solution as a globular entity, the thermodynamic parameters of which compare well with those of similar domains and, in general, with those of globular proteins.

Kinetic Properties of ADA2h. From the kinetic point of view ADA2h folds very rapidly. Previous studies showed that this region is indispensable for the folding of the whole proenzyme, *in vivo* as well as *in vitro* (Avilés *et al.*, unpublished results). In short, the depletion of the activation segment by limited proteolysis or by protein engineering has shown that the resulting carboxypeptidase does not fold in the broad conditions assayed. Similar observations have been made for the pro-segments of other proteins (Winther & Sørensen, 1991; Baker et *al.*, 1992; Sørensen *et al.*, 1993). Taking all of these considerations together with the fact that, as commented above, carboxypeptidases cannot reach a proper folding without the activation domain, it is reasonable to hypothesize that *in vivo* the region of procarboxypeptidase A2 corresponding to ADA2h folds very quickly, and that the folding of the carboxypeptidase moiety does not occur until ADA2h is already folded. Perhaps, in the folding pathway of procarboxypeptidases one of the possible intermediates is the one that has the activation domain already folded but not the enzyme moiety. Although further studies must be done to check out this hypothesis, the role of the folded activation domain would be that of lowering the energy of the transition state in the folding pathway of the complete proenzyme.

ADA2h Folding. There are several criteria that need to be fulfilled by a protein to consider that it follows a two-state transition. First of all, the unfolding of the protein by a denaturant agent must fit to a single transition curve. This criterion does not eliminate the possibility of having equilibrium and kinetic intermediates, and, consequently, it is the least stringent of all. Second, the differential scanning calorimetry unfolding endotherm must fit the $C_p(T)$ function corresponding to a two-state model. If the protein fulfills this criterion, the existence of a significant population of stable intermediates in equilibrium can be discarded. Finally, the most sensitive evidence for the detection of transient intermediates is the fitting of the logarithm of the rate constants of unfolding and refolding to two linear equations, from which we should obtain identical ΔG_{H_2O} and m values, within experimental error, to those obtained by equilibrium denaturation (Jackson & Fersht, 1991).

Thermal unfolding of the domain analyzed here can be described very well in equilibrium by a monomolecular two-state model at pH 7.0, 50 mM phosphate. The ratio between the van't Hoff and the calorimetric enthalpy in the conditions used in the equilibrium and kinetic analyses is, within the limits of experimental error, very close to the value observed for the two-state denaturation of several globular proteins (1.00 ± 0.05; Privalov, 1979). On the other hand, at lower ionic strength and/or at higher pH values, there is calorimetric evidence for the association, to some extent, of the folded domain in solution prior to its thermal unfolding to give unfolded monomers. The same phenomenon has been observed in other proteins (Filimonov *et al.*, 1993).

It is now widely accepted that there must be kinetic intermediates in the folding pathway of proteins [for a review, see Kim and Baldwin (1990)]. In most of the monomeric proteins described so far in the literature, a significant deviation from linearity has been found in the refolding reaction which is attributed to the accumulation of a folding intermediate (Ikeguchi *et al.*, 1986; Kuwajima *et al.*, 1988; Matouschek *et al.*, 1992; Schreiber & Fersht, 1993; Muñoz *et al.*, 1994). However, in small monomeric proteins with sizes close to ADA2h, the CI2 chymotrypsin inhibitor (Jackson & Fersht, 1991), the G-domain (Alexander *et al.*, 1992a), the SH3-domain of α-spectrin (Viguera *et al.*, 1994), and the CspB protein (Schindler *et al.*, 1995), no deviation from linearity was found, and the slow phases detected were due to a small proportion of the molecules that in the unfolded state have *cis*-prolyl bonds. The same phenomenon has been found for ADA2h. The molecules with the prolyl bonds in the *trans* conformation fold very rapidly and do not exhibit a significant deviation from linearity. A small proportion of the molecules fold slowly with a rate constant, independent of urea concentration, whose value is similar to those found for *cis-trans* proline isomerization processes

Table 1: Thermodynamic Unfolding Values of ADA2h Obtained from Chemical Denaturation, Differential Scanning Calorimetry, and Kinetics, at pH 7.0 and 298 K

method	ΔG_{H_2O} (kJ mol^{-1})	m (kJ mol^{-1} M^{-1})	T_m (°C)	$\Delta H_u(T_m)$ (kJ mol^{-1})
equilibrium	17.0 ± 1.0	4.0 ± 0.2		
kinetics	17.9 ± 0.7	4.22 ± 0.3		
DSC	15.1 ± 2		77.0	199

(Jackson & Fersht, 1991; Matouschek *et al.*, 1992; Viguera *et al.*, 1994). In a strict sense, this slow phase could be considered as due to folding intermediates, although the intermediates we are actually seeking are those accumulating in the folding reaction of unfolded proteins with all the prolines in the native conformation (Matouschek *et al.*, 1992; Schreiber & Fersht, 1993; Muñoz *et al.*, 1994). Our results clearly demonstrate that such intermediates do not accumulate in the refolding reaction of ADA2h and that kinetic and equilibrium data render similar values for ΔG_{H_2O} and m (Table 1).

What the acyl-coenzyme A binding protein CI2, G-domain, SH3-domain, CspB protein, and ADA2h have in common is that they have a very small core within the limits of what has been postulated to be a stable folding domain without disulfide bridges (Privalov & Gill, 1988). The acyl-coenzyme A is an all α-protein, the SH3-domain of α-spectrin and the CspB protein are β-sheet barrel proteins, while CI2, the G-domain, and ADA2h belong to the α+β group of proteins, and, in consequence, the lack of any putative stable folding intermediates is not due to any kind of secondary structure restriction. The only common point among these six proteins is their small size (<90 aa).

In general, it seems that the accumulation of kinetic intermediates during the folding pathway is imposed by the existence of one or more large hydrophobic cores, or two or more subdomains, that slow down the acquisition of the tertiary structure. In contrast, when the size of the protein does not require such a degree of complexity, the acquisition of the secondary structure will be simultaneous with that of the tertiary structure.

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